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Title: The Inhibitory Activity of Essential Oil Constituents Against *Clostridium perfringens* Type A Isolates.

Abstract approved:

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Clostridium perfringens is a gram-positive spore forming bacterium that commonly found in the environment and in the gastrointestinal (GI) tract of human and other animals where it is a part of the normal flora. It is also known to be a pathogenic bacterium in human and domestic animals. Among five types (A-E), type A strains of *C. perfringens* are considered as the main causative agent of *C. perfringens*-associated GI diseases in human such as, food poisoning (FP) and non-food-born (NFB) GI diseases. Several intrinsic and extrinsic factors contribute to the pathogenesis of *C. perfringens* type A strains. First intrinsic factor is spore; in addition to spore ubiquitousness, it is highly resistant to various inactivation factors such as, moist and dry heat, UV radiation, nitrate and some toxic chemicals. Second important intrinsic factor is *Clostridium perfringens* enterotoxin (CPE), which is produced by *C. perfringens* type A strains and proved to be an essential virulence factor for *C. perfringens* pathogenesis. On the other hand, there are some external factors involved in pathogenesis of *C. perfringens* such as, improper cooking,

inadequate cooling, unsuitable storage temperature of meat and poultry products. By using preservation method such as thermal processing, the risk of having food-borne diseases is decreased, however, new and alternative strategies are in urgent demand specially, against *C. perfringens* since it produce extremely heat resistant spores.

In this work, the efficacy of essential oils constituents (cinnamaldehyde, eugenol, carvacrol, and allyl isothiocyanate AITC) towards inhibiting *C. perfringens* FP and NFB isolates have been determined in laboratory conditions and chicken meat model. In laboratory medium, lower concentration (0.05%) of cinnamaldehyde, eugenol, carvacrol showed noticeable inhibition in spore germination. AITC at 0.05% and 0.1% exhibited no inhibition. Furthermore, all tested essential oils constituents at 0.05 - 0.1% illustrated significant inhibitions in spore outgrowth and vegetative growth of *C. perfringens*. However, most significant inhibition was detected in the presence of AITC or carvacrol at 0.05% concentration. In meat model system, AITC at various concentrations (0.5%, 1%, 1.5%, and 2%) were able to inhibit the growth of *C. pefringens* FP and NFB isolates, but no inhibitory activity of cinnamaldehyde, eugenol, and carvacrol was found at the same concentrations. Even at higher concentration (5%), carvacrol showed no antimicrobial activity against *C. pefringens* FP and NFB isolates in chicken meat model.

Collectively, our current work contributes to food industry in order to control *C. perfringens* spore and vegetative cells of both FP and NFB isolates in laboratory condition and chicken meat model system. The antimicrobial agents used in this study

are generally recognized as a safe (GRAS) agents to food formulation. Combination of these components should be an active method to control the risk of *C. perfringens* FP and NFB diseases.

The Inhibitory Activity of Essential Oil Constituents Against *Clostridium perfringens* Type A Isolates.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Saud Alanazi, Author

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CONTRIBUTION OF AUTHORS

Chapter 2. Dr. Maryam Alnoman and Dr. Mahfuzur Sarker were involved with experimental design and manuscript preparation.

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Chapter 1

General introduction and Literature Review

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium of the genus Clostridium; which involves more than 100 species that include free-living bacteria, as will as important pathogens (Labbe & Tang, 1983; Li et al., 2013; Bruce A. McClane, 2007). The main species responsible for diseases in humans are *C. botulinum, C. tetani* and *C. difficile*, and some have industrial benefits such as *C. thermocellum* and *C. acetobutylicum* (Hatheway, 1990). *C. perfringens* is a ubiquitous microorganism found naturally in soil, water, milk, dust, and wastewater. Also, it is part of the normal intestinal flora of human and animals (B. A. McClane & Chakrabarti, 2004; Smedley, Fisher, Sayeed, Chakrabarti, & McClane, 2004). *C. perfringens* is a causative agent of many diseases including, gas gangrene, necrotic enteritis, septicemia cellulitis, *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) diseases (Awad & Rood, 1997). FP is currently ranked as the second most commonly reported bacterial food-borne disease in the United States with 250,000 cases per year (Mead et al., 1999). The optimum growth temperature of *C. perfringens* is 35 ^{o}C - 40 ^{o}C (Cordoba, Aranda, Medina, Jordano, & Cordoba, 2001). The optimum pH for growth is between pH 6.0-7.0, which is the pH range of many cooked meat and poultry products.

Growth is possible under pH 5.0 and more than pH 8.0. The minimum water activity for *C. perfringens* growth is 0.93-0.97 depending on the solute (B. A. McClane, 1996; Novak, Tunick, & Juneja, 2001). Several factors have been attributed to the pathogenicity of *C. perfringens*. First, its ability to produce a subset of ~17 different toxins; combination of some toxins permits the bacteria to cause different diseases (McDonel, 1980). *C. perfringens* can be categorized into five different toxinotypes (A-E) depending on the production of the four major lethal toxins (alpha, beta, iota and epsilon) (Raju & Sarker, 2005; Simpson, Stiles, Zepeda, & Wilkins, 1989). Second, *C. perfringens* has generation time of less than 10 min, which makes it easy to cause food contamination. Another important factor in the pathogenesis of *C. perfringens* is the survival of *C. perfringens* spores under harsh conditions such as heat, dry and some toxic chemicals (Huang & Sarker, 2006; Sarker, Shivers, Sparks, Juneja, & McClane, 2000).

1.1. *C. perfringens* Major toxins

1.1.1. Alpha Toxin

All types of *C. perfringens* strains produce alpha toxin, but type A attributed as the highest producer (Czeczulin, Collie, & McClane, 1996; B. A. McClane, 1996; McDonel, 1980). The alpha toxin gene *plc* is located on the chromosome of *C. perfringens.* VirR/VirS, a two component regulatory system, is known to regulate the expression of the *plc* gene (Ohtani, Bhowmik, Hayashi, & Shimizu, 2002). This 43kDa toxin contains tow domains, an N-terminal domain, and a C-terminal domain (Sakurai, Nagahama, & Oda, 2004; Uzal et al., 2014). The N-terminal domain is a zinc metalloprotease that has both phospholipase C and sphingomyelinase activity which catalyze the cleavage of the polar head group resulting in a charged head group and a diacylglycerol group or ceramide, respectively (Krug & Kent, 1984; Titball, 1993; Young, Snyder, & McMahon, 1991). The C-terminal domain is a calcium binding domain. *plc* in *C. perfringens* has two functions. The first one is to degrade lecithin found in the eukaryotic cell membranes. The second function is to degrade sphingomyelin that is also present in the membrane of eukaryotic cells (Nagahama, Michiue, & Sakurai, 1996; Nagahama et al., 2007).

Alpha toxin is the toxin that involved in *C. perfringens* gas gangrene and Clostridial myonecrosis, which is a histotoxic infection of humans and animals (Flores-Diaz & Alape-Giron, 2003; Rood & Cole, 1991). The disease developed by the infection of wound and associated with two events. First, bacteria must be imported into soft tissues, often following a traumatic injury, which contains the ingress of soil or other organic matter containing *C. perfringens.* Secondly, since *C. perfringens* is an anaerobe bacterium, the blood supply to the infected tissues must be impaired in some way so the bacteria can grow. Clinical signs of gas gangrene can come out as soon as 6 h after trauma and once the contaminated wounds lack the oxygen due to disruption of the arterial blood supply (Flores-Diaz & Alape-Giron, 2003). Once the diseases initiated, it spreads very quickly through healthy tissues, leading to shock and death if not treated.

1.1.2. Beta Toxin

Beta toxin can be produced extracellularly by two different types of *C. perfringens,* which are type B and type C isolates (Songer, 1996). It is a major pathogenic determinant of necrotic enteritis, and enterotoxaemia in human and many domesticated livestock, such as, sheep, lamb, fowl and pigs (Itodo, Adesiyun, Adekeye, & Umoh, 1986). The beta-toxin has molecular weight around 40 kDa and the toxin gene, *cbp*, is carried on a large plasmid (Hatheway, 1990). This toxin can be

lethal by causing hemorrhagic necrosis leading to significant destruction of intestinal villi (Rood & Cole, 1991). However, inactivation of beta toxin takes place in human gastrointestinal tract due to the normal existence of some enzymes such as trypsin. Pig Bel and enteritis necroticans are usually correlated with high consumption of protein meal by individuals who have lower pancreatic enzymes such as trypsin (Lawrence, 1979).

1.1.3. Epsilon Toxin

The major virulence factor of *C. perfringens* type B and type D isolate is Epsilon toxin. It is a pore forming toxin and encoded by *etx* on large plasmid (Katayama, Dupuy, Daube, China, & Cole, 1996). It is the third most potent toxin classified as B bioterrorist agent after tetanus and botulinum neurotoxins (Petit et al., 1997). Epsilon toxin is produced as inactive toxin, but it becomes active either by the enzymes in the gastrointestinal tract or by the *C. perfringens* lambda toxin.

1.1.4. Iota Toxin

Iota toxin is known to cause sporadic diarrhea in domesticated cattle (Barth, Aktories, Popoff, & Stiles, 2004). This toxin is produced by *C. perfringens* type E strains. The genes of Iota toxin are *iap* and *ibp,* both are located in the plasmid and each one has different function. Ib is a binding component that bind to the cells and

translocates the Ia into the cytosol, where Ia is an enzyme component (Simpson, Stiles, Zepeda, & Wilkins, 1987).

1.1.5. *Clostridium perfringens* Enterotoxin (CPE)

C. perfringens enterotoxin is a causative agents of several gastrointestinal (GI) diseases caused by *C. perfringens* type A isolates, including FP and NFB GI illnesses such as antibiotic associated diarrhea and sporadic diarrhea (Sarker, Carman, & McClane, 1999). CPE is a heat and pH labile protein with the molecular mass of 35-kDa, and about 5 % of type A isolate produces CPE (B. A. McClane & Chakrabarti, 2004; Sarker et al., 1999). It has been shown that the *cpe* gene is located on the chromosome of FP strains and on the plasmid of NFB isolates (Collie & McClane, 1998).

CPE protein is known to be a single polypeptide that has 391 amino acids, and consists of tow domains. The first domain is C-terminal CPE receptor-binding domain, although this domain binds tightly to receptors, early studies showed half of the C-terminal lacks cytotoxic activity (Freedman, Shrestha, & McClane, 2016; B. A. McClane & Chakrabarti, 2004). The second domain is N-terminal oligomerization and pore formation domain, it is about 80 -106 of alternating hydrophobic and hydrophilic amino acids (Briggs et al., 2011; Saitoh et al., 2015).

CPE production and regulation are similar to other *Clostridium spp* and *Bacillus spp*. They are strongly dependent on global regulators and sporulation-specific sigma factors (Freedman et al., 2016; Harry, Zhou, Kroos, & Melville, 2009; Li & McClane, 2010). During the sporulation of *C. perfringens*, the master sporulation regulator Spo0A is activated by unknown signal. This signal could be the presence of bile salt or inorganic phosphate as previous reports in *Bacillus* and *Clostridium spp* showed that the activity of Spo0A is dependent on phosphorylation. Phosphorylated Spo0A binds to the genes that are responsible for sporulation, once bound; the expression of these sporulation genes will be activated (Talukdar, Olguin-Araneda, Alnoman, Paredes-Sabja, & Sarker, 2015). In *C. perfringens*, SigF is one of the target genes that are likely to be activated by phosphorylated *Spo0*A (Huang, Waters, Grau, & Sarker, 2004; Li & McClane, 2010). It is an alternative sigma factor that is necessary for CPE production and *C. perfringens* sporulation; also, it regulates other sporulation-specific sigma factors including SigG, SigE, and SigK (Li & McClane, 2010). All of these sigma factors are important for the sporulation of *C. perfringens*. However, SigK and SigE, but not SigG are required for the CPE production (Li & McClane, 2010). The expression of *cpe* gene is regulated by three promoters upstream of the chromosomal or the plasmid *cpe* gene (Zhao & Melville, 1998). Promoter (P1) demonstrates similarity to recognition sequence for SigK, while promoter (P2) and promoter (P3) contain sequence similar to SigE recognition sequence (Freedman et al., 2016; Talukdar et al., 2015; Zhao & Melville, 1998).

The initiation of CPE cytotoxic activity is required by the binding of CPE to claudin receptors on the surface of the host cells (Gunzel & Yu, 2013). Once bound; it will create small complex that has ability to form a complex on the surface of the plasma membrane (Robertson et al., 2007). This small complex will bind to different protein to form large complex that can stimulate the permeability of the plasma membrane and cause calcium influx (Li & McClane, 2006). Basically, the severity of diseases depends on the amount of CPE, small amount of CPE causes a few number of pores with some calcium influx that leads to apoptosis (Chakrabarti & McClane, 2005). However, a high dose of CPE causes a significant calcium influx and results in necrotic cell death and damage to the small intestine (Chakrabarti, Zhou, & McClane, 2003).

1.2 Bacterial Spore Germination

C. perfringens spores are known to be dormant and resistant under harsh conditions such as heat, dry, and chemicals. However, spores lose their dormancy and germinated under favorable growth conditions (Setlow, 2014). The initiation of germination process can be done by variety of germinates such as nutrient, 1:1 chelate of Ca^{2+} and DPA, and the cationic surfactant dodecylamine (Olguin-Araneda, Banawas, Sarker, & Paredes-Sabja, 2015). The complete spore germination process can be divided into two stages, stage I and stage II (Setlow, 2014). In stage I, the outer layer proteins of the spores facilitate the movement of germinant molecules. Then, an interaction between germinant molecules and germinant receptor (GR) take

place in the spore's inner membrane IM. As a result, the spore's IM become more permeable and replace water with different cations in the spore's core such as monovalent cations and dipicolinic acid (DPA) chelated with divalent cations, primarily Ca^{2+}(Moir, 2006; Setlow, 2014). In stage II, the cortex peptidoglycan hydrolyze and more water absorbe in the germinating spore, and then all metabolic activity resume and eventually the spores germinate. The spore germination process is followed by the spore outgrowth and return to actively growing cell (Moir, 2006; Olguin-Araneda et al., 2015; Setlow, 2014).

Unlike *C. perfringens* and other *Bacillus spp*, *Bacillus subtilis* spores germination is fully understood (Moir, Corfe, & Behravan, 2002; Paidhungat & Setlow, 2000; Paredes-Sabja, Setlow, & Sarker, 2009; Paredes-Sabja, Udompijitkul, & Sarker, 2009). In spore's inner membrane of *B. subtilis,* three major GRs termed *GerA, GerB*, and *GerK,* encoded by tricistronic operons that are expressed in late stage of sporulation. Loss in these tricistronic GRs operons will cause a function loss in that GR (Pelczar & Setlow, 2008). In *C. perfringens* spores, the GR has been studied and the genes organization was different than that in *B. subtilis* (Paredes-Sabja, Setlow, & Sarker, 2011; Paredes-Sabja, Torres, Setlow, & Sarker, 2008). Instead of the tricistonic GR, there is a monocistronic *gerAA* located a bit far from *gerK* locus. The *gerK* locus contains *gerKA*, *gerKB* and *gerKC*; *gerKB* is a monocitronic gene, but *gerKA* and *gerKC* are organized as a bicistronic operon that has opposite orientation compared to *gerKB*. Our laboratory has shown that GerKA

and/or GerKC are mandatory GR proteins for normal spore germination of *C. perfringens* in the present of germinants such as KCL, L-asparagine, or L-asparagine-KCL mixture (AK) (Paredes-Sabja et al., 2008; Paredes-Sabja, Udompijitkul, et al., 2009; Udompijitkul, Alnoman, Banawas, Paredes-Sabja, & Sarker, 2014). Our recent finding have shown that GerKC is the major GR protein for *C. perfringens* spore germination (Banawas et al., 2013).

1.3 *C. perfringens* inactivation

Inactivation of *C. perfringens* spores and vegetative cells is one of the major concerns in food industry (Novak et al., 2001). *C. perfringens* spores and vegetative cells are resistant to several inactivation strategies. Since it is ubiquitous, spores can be found in food processing facilities that have the ability to survive various stress factors such as high temperature, pressure, and chemicals (B. A. McClane, 1996). Spores of *C. perfringens* were also resistant to preservative and inactivation treatment such as low temperature, osmosis-induce stress, drying, and nitrite (Labbe & Tang, 1983; Sarker et al., 2000). Even though the vegetative cells of *C. perfringens* are less resistant to those stress factors, it can grow rapidly under wide range of temperature and thus cause diseases (B. A. McClane, 1996; Wells-Bennik et al., 2016). Strategies to inactivate *C. perfringens* spores and vegetative cells are demanded in food industries (Novak et al., 2001; Wells-Bennik et al., 2016).

1.3.1 Essential oils

Recently, a considerable effort has been applied by clinical research towards the expansion of new, active and safe antimicrobials against bacterial infection. Instead of heat processing technologies, using natural antimicrobial compounds is an alternative strategy as antibacterial, antiviral, and antifungal agents. Also, they extend the shelf life of the food and improve its quality (Oussalah, Caillet, Saucier, & Lacroix, 2006; Smith-Palmer, Stewart, & Fyfe, 1998). Essential oils are volatile and aromatic liquids extracted from plant materials (Schnitzler & Reichling, 2011; Smith-Palmer et al., 1998). Essential oils are secondary metabolite, which plays a role in plant defense and possess antimicrobial properties. According to the Food and Drug Administration, most essential oils are generally recognized as a safe (GRAS) (Hyldgaard, Mygind, & Meyer, 2012). Recently, various essential oils and their constituents exhibited significant inhibition on the growth of spoilage and pathogenic microorganisms on food (Cha & Chinnan, 2004; Gutierrez, Barry-Ryan, & Bourke, 2008; Natrajan & Sheldon, 2000). In some cases, using one or more principle constituents instead of essential oils may provide equal antimicrobial activity with milder flavoring attributes (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011). Cinnamaldehyde, eugenol, carvacrol, and allyl isothiocyanate (AITC) are active components of oil extracts. Cinnamaldehyde, Eugenol and (AITC) have been reported to inhibit the growth of *Clostridium botulinum, Staphylococcus aureus, Escherichia coli* 0157: H7 and *Salmonella enterica* serovar Typhimureum (Blaszyk & Holley, 1998; Burt, 2004; Cosentino et al., 1999). Carvacrol has been well examined for its

antimicrobial activity against many food-borne pathogens such as *E. coli, Listeria monocytogenes, Bacillus cereus, Salmonella spp*, and *Lactobacillus sakei*.(Ultee, Bennik, & Moezelaar, 2002; Ultee, Kets, & Smid, 1999) Recently, a study determined the activity of carvacrol, eugenol, and thyme essential oils against *salmonella Enteritidis, S. Heidelberg, S. Typhimurium* on turkey breast cutlets and revealed the sensitivity of the bacteria to these essential oils with higher activity to carvacrol compared with other plant essential oils (Nair, Nannapaneni, Kiess, Schilling, & Sharma, 2014).

1.4 Objective of this study:

In this study we aimed to evaluate:

(1) the antimicrobial activity of Cinnamaldehyde, Eugenol, Allyl isothiocyanate (AITC), and Carvacrol against the spore germination, outgrowth and vegetative growth of *C. perfringens* FP and NFB isolates, and

(2) the effectiveness of these essential oil constituents to control *C. perfringens* growth in chicken meat products.

Chapter 2

The Inhibitory Activity of Essential Oil Constituents Against *Clostridium perfringens* Type A Isolates.

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Abstract

C. perfringens type A isolates are the causative agents of *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) human gastrointestinal diseases. In this study, we evaluated the antimicrobial effect of essential oil constituents (cinnamaldehyde, eugenol, allyl isothiocyanate (AITC), and carvacrol) against *C. perfringens* FP and NFB isolates grown in laboratory medium and chicken meat. Lower concentration (0.05%) of cinnamaldehyde, eugenol, carvacrol, but not AITC showed noticeable inhibition in spore germination. Furthermore, all tested essential oil constituents at 0.05-0.1% illustrated significant inhibitions in spore outgrowth and vegetative growth of *C. perfringens*. However, most significant inhibition was detected in the presence of AITC or carvacrol at 0.05% concentration. In meat model system, AITC at various concentrations (0.5%, 1%, 1.5%, and 2%) were able to inhibit the growth of *C. pefringens* FP and NFB isolates, but no inhibitory activity of cinnamaldehyde, eugenol, and carvacrol was found at the same concentrations. Even at higher concentration (5%), carvacrol showed no antimicrobial activity against *C. pefringens* FP and NFB isolates in chicken meat model. Collectively, although all constituents observed inhibitory effects against spore outgrowth and vegetative cells of *C. perfringens* FP and NFB isolates in laboratory condition, only AITC was able to control *C. perfringens* spores into meat model system.

1. Introduction

Clostridium perfringens is a gram-positive, rod-shaped, anaerobic, endospore forming bacterium, which is a causative agent of histotoxic and gastrointestinal (GI) diseases in human and animal. *C. perfringens* are classified into 5 types A-E (Bruce A. McClane, 2007; Petit, Gibert, & Popoff, 1999). These types are established based on the 4 major toxin production (alpha, beta, epsilon, and iota) (Bruce A. McClane, 2007; Petit et al., 1999). A small group (~ 5%) of type A isolates, that produces *C. perfringens* enterotoxin (CPE), is causing *C. perfringens* type A food poisoning (FP) as will as nonfood-borne NFB human gastrointestinal (GI) illnesses such as nosocomial diarrheal diseases, sporadic diarrhea, and antibiotic associated diarrhea. The CPE-encoding gene (*cpe*) has been shown to be located on the chromosome or a large plasmid of *C. perfringens* FP and NFB isolates, respectively (Collie & McClane, 1998; Sarker et al., 2000). *C. perfringens* type A food poisoning currently ranks as the second most commonly reported bacterial food-borne (FP) illnes in the United States accounting for about one mllion cases per year (Hoffmann, Batz, & Morris, 2012; Juneja & Thippareddi, 2004; Lynch et al., 2006).

C. perfringens vegetative cells can easily be killed by applying thermal treatment, however, the spores of this bacterium survive heat treatment and could be activated to germinate, outgrow and multiply onto food products to a risky level (Li & McClane, 2006; Paredes-Sabja et al., 2008; Sarker et al., 2000; Thippareddi, Juneja, Phebus, Marsden, & Kastner, 2003). One of the main goals of the food industry is to develop bacterial spore-inactivation strategies alternative to heat

processing technologies, that meet consumer demand for ensured food safety, extended shelf life, and enhanced food quality. Previous studies in our lab demonstrated the inhibitory effects of polyphosphates, nisin, sorbate benzoate, and chitosan (Akhtar, Paredes-Sabja, & Sarker, 2008; Alnoman, Udompijitkul, Paredes-Sabja, & Sarker, 2015; Alnoman, Udompijitkul, & Sarker, 2017; Udompijitkul, Paredes-Sabja, & Sarker, 2012). In laboratory condition, these antimicrobial agents exhibited slight inhibition on spore germination and significant inhibition on spore outgrowth and vegetative growth of *C. perfringens* isolates. However, in meat model system, only polyphosphates and chitosan exhibited inhibitory activity against spore germination and outgrowth of *C. perfringens* isolates (Akhtar et al., 2008; Alnoman et al., 2017). Alternative strategies consider the use of natural antimicrobial compounds in foods such as spices and herbs (Sabah, Thippareddi, Marsden, & Fung, 2003; Thippareddi et al., 2003; Valenzuela-Martinez et al., 2010). Essential oil constituents (EOC) are natural, aromatic and volatile liquid extracted from plant material such as flowers, roots, seeds, leaves, peel and whole plant (Hyldgaard et al., 2012; Kuorwel et al., 2011). It is important for plant defense and considered to be secondary metabolites (Herman, Herman, Domagalska, & Mlynarczyk, 2013). Many components of essential oils have been accepted by the European Commisson as flavoring agents in food products. Such as linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene. Also, in United States, Food and Drug Adminstration (FDA) considers these components as generally

recognized as a safe (GRAS) and other essential oils such as clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon (Hyldgaard et al., 2012). Recently, extensive research has been reported on the use of EOC as antimicrobial agents against different spoilage bacteria (Burt, 2004; Oussalah et al., 2006; Smith-Palmer et al., 1998; Ultee et al., 2002). Their inhibitory activity has been assigned to a number of substituted aromatic molecules such as cinnamaldehyde, eugenol, allyl isothiocyanate (AITC), and carvacrol (Burt, 2004; Cha & Chinnan, 2004; Gutierrez et al., 2008; Kuorwel et al., 2011; Natrajan & Sheldon, 2000). Cinnamaldehyde, eugenol and AITC have been reported to inhibit the growth of *Clostridium botulinum, Staphylococcus aureus, Escherichia coli* 0157: H7 and *Salmonella enterica* serovar Typhimureum (Blaszyk & Holley, 1998; Burt, 2004; Cosentino et al., 1999). Carvacrol has been well examined for its antimicrobial activity against many food-borne pathogens such as *E. coli, Listeria monocytogenes, Bacillus cereus, Salmonella spp,* and *Lactobacillus sakei* (Nair et al., 2014; Natrajan & Sheldon, 2000; Smith-Palmer et al., 1998).

Although extensive research has been focused on the antimicrobial activity of plant EOC against various food-borne pathogens, the information on the inhibitory effect of EOC against *C. perfringens* is limited. Therefore, the objectives of this study were to evaluate (1) the antimicrobial activity of cinnamaldehyde, eugenol, allyl isothiocyanate (AITC), and carvacrol against the spore germination and outgrowth of

C. perfringens FP and NFB isolates, and (2) evaluate the inhibitory activity of these EOC in germination and outgrowth of *C. perfringens* spores in chicken meat products.

2. Material and methods:

2.1. Bacterial strains and growth conditions

Six strains of enterotoxigenic *C. perfringens* type A were used in this study including three FP isolates (SM101, E13, and NCTC8239), and three NFB isolates (F4969, B40, and NB16) (Sarker et al., 2000). Cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) was used to maintain isolates in stock cultures stored at -20 °C. The bacterial growth was revieved by inoculating from cooked meat cultures into fluid thioglycollate medium (FTG) (Difco) then incubating overnight at 37 °C. TGY broth (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) was used for vegetative growth (Paredes-Sabja et al., 2008) .

2.2. Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008). Briefly, 0.1 ml from *C. perfringens* stock culture was inoculated into 10 ml FTG and incubated overnight at 37 °C. Then, 0.4 ml of the overnight culture were transferred to new FTG medium and grew for 8 h at 37 °C. Next, 0.4 ml of the 12 h FTG culture were transferred into Duncan-Strong Sporulating medium (1.5% protease peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic (Na_2HPO_4) (andydrous), 0.4% soluble starch) (Duncan & Strong, 1968) and incubated overnight at 37 °C. The maximum amount of spores was confirmed by using phase contrast microscope

(Leica MDLS, Leica microsystems). Large amounts of spores were gained by scaling up the latter procedure. Repeated washing and centerifuging with cold sterile distilled water was performed to purify spore cultures. After obtaining > 99% free spores, purified spores were suspended in sterile distilled water and adjusted to a final optical density at 600 nm (OD600) of ~ 6.0 that corresponds to approximately 10^8 CFU/ml using SmartspecTM 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), and stored at -20 °C until used (Paredes- Sabja et al., 2008).

2.3. EOC used as antimicrobials in this study

EOC used in this study were: cinnamaldehyde, eogenol, allyl isothiocyanate (AITC) and carvacrol. Cinnamaldehyde (M_W 132.16 g/mol), eugenol (M_W 164.21 g/mol), and AITC (M_W 99.16 g/mol) were purchased from Alfa-Aesar (Sunrise Valley Drive Reston, VA United States). Carvacrol (M_W 150.22 g/mol) was purchased from TCI America (9211 N Harborgate St, OR United States). EOC were added directly to sterile 10 ml TGY (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine).

2.4. **C. perfringens** *spore germination in the presence of EOC*

The purified spore suspensions were heat activated at 80 °C for FP spores and at 75 °C for 15 min for NFB spores as described previously (Paredes-Sabja et al., 2008; Paredes-Sabja, Udompijitkul, et al., 2009), and then cooled in water bath at room

temperature for 5 min. The germination of spore was measured by mixing heat-activated 33 µl of spore suspension with 167 µl of the fresh TGY supplemented with various concentrations of oils in 96-will microtiter plate and incubated at 37 °C for 60 min and then OD_{600} was monitored by the spectrophotometer (BioTek Insrtrument Inc., Winooski, VT). Spore germination was also confirmed by phase contrast microscopy, in which spore's refractility will be lost and become phase dark spores upon germination. Germination rate was revealed as percentage fall of OD_{600} relative to the initial value after 60 min of incubation in TGY alone or in the presence of essential oil constituents. Germination inhibition was expressed as percent inhibition of spore germination compared with the control germination (without essential oils), i.e., a percentage of inhibiton = (OD_{600} increase in treatment/ OD_{600} increase in control) X 100 (Alnoman et al., 2015; Cortezzo, Setlow, & Setlow, 2004). All experiments were performed in triplicate with different spore preparations for each strain.

2.5. Outgrowth of C. perfringens *spores in the presence of EOC*

The spore outgrowth was tested in a fresh TGY medium. Briefly, 300 µl of adjusted spore at an OD_{600} of ~ 6.0 were heat activated (at 80 °C for 10 min for FP spores and at 75 °C for 15 min for NFB spores), cooled in water bath at room temperature for 5 min, then inoculated into fresh 10 ml TGY broth alone (control) or supplemented with different concentration of essential oil constituents . After that, all

cultures incubated at 37 °C and the OD_{600} was measured over a time interval for up to 180 min post-inoculation.. Spore outgrowth rates were calculated as percent increase in OD600 , and a percentage of inhibiton = (OD_{600} increase in treatment/ OD_{600} increase in control) X 100 (Cortezzo et al., 2004). The experiments were achieved in triplicate with different spore preparations (Alnoman et al., 2015; Paredes-Sabja, Udompijitkul, et al., 2009).

2.6. Vegetative growth of C. perfringens *isolates in the presence of EOC*

The inhibitory activities of essential oil constituents on the growth of *C. perfringens* were monitored in a fresh TGY medium. FTG growth culture of each *C. perfringens* strain was incubated overnight. Then, 400 µl was transferred into 10 ml of TGY and grown for 3 h at 37 °C. Next, 400 µl of 3-h TGY culture was then transferred into 10 ml of TGY alone (control) or supplemented with various concentrations of EOC. For monitoring the vegetative growth OD_{600} of inculated TGY broth was measured at various time points for up to 24 hours. Experiments were achieved in triplicate.

2.7. C. perfringens *spores germination and outgrowth in cooked chicken meat in the presence of EOC*

The chicken meat was purchased from a local supermarket in Corvallis, OR., U.S.A, and ground immediately in a sterile stainless steel blender. (10 g/bag) was weighed for each meat samples and then placed into sterile plastic bag (5.5" W × 6"L) (SealaMeal vacuum storage bag, Sunmeam Products, Inc., Boca Raton, Fla., U.S.A.), and then sealed and stored at - 20 until use (Alnoman et al., 2015).

The activity of essential oil constituents on the growth *of C. perfringens* spores in chicken meat was performed by mixing either FP isolates (SM101, NCTC8239, and E13) or NFB isolates (F4969, B40, and NB16) (Udompijitkul et al., 2012).

Before each experiment, the 10 g meat sample thawed at room temperature and then adding100 µl of the spore cocktail of *C. perfringens* FP or NFB isolates prepared as described above and then adding the desired dose of essential oil constituents. After resealing the bag, spores and essential oils were distributed by a manual massage for 1 min. Samples were cooked at 80 ^{o}C for 20 min and then cooled down in the water bath at room temperature for 10 min (Akhtar et al., 2008). To ensure that meat samples are free of naturally occurring bacteria, negative control were performed in each replicate. Two bags were used in each treatment. One bag to define the initial number of *C. perfringens*, the other meat sample was transferred to a sterile Petri dish and incubated at 37 ^{o}C under anaerobic condition for 6 h. The population of *C. perfringens* was identified by transferred the 10 g meat sample to a stomacher bag, and then adding 90 ml 0.1% (w/v) peptone water, and serially diluted in 25 mM sodium phosphate buffer, and then plated in anaerobic condition at 37 °C for 24 h

onto BHI agar plates. Results were expressed as CFU/g (Alnoman et al., 2015; Udompijitkul et al., 2012).

2.8. Statistical analysis

For statistical significant, tow-tailed student's t-test with 95% confidence intervals were used. The comparisons were made by using Prism version 7 software (GraphPad).

3. Results and discussion

3.1. The inhibitory activity of EOC on C. perfringens *spore germination*

As expected from previous studies (Akhtar et al., 2008; Alnoman et al., 2015; Alnoman et al., 2017; Udompijitkul et al., 2012), spores of all tested clinical *C. perfringens* FP and NFB isolates were gereminated significantly in TGY after 60 min incubation at at 37 ^{o}C (data not shown). When similar gemination assay was performed with TGY supplemented with EOC, cinnamaldehyde, eugenol, and carvacrol inhibited the germination capabilities of spores of most tested FP and NFB isolates. However, under similar condition AITC did not show any inhibition of spore germination (Table 1). Although 0.1% is the most effective inhibitory concentration for cinnamaldehyde, eugenol, and carvacrol against all tested strains, 0.05 % showed significant inhibitory effect against spores of two NFB strains (F4969 and NB16) and moderate effects against spores of FP strains (Table 1). Among the six, F4969 spores was the most sensitive to cinnamaldehyde, eugenol, and carvacrol, however, SM101 and B40 illustrated some resistance to all tested EOC. Collectively, these results indicate that the inhibition of spore germination in TGY is dependent on strains and type and concentration of EOC. This is consistent with our previous reports where the inhibition of *C. perfringens* spore germination was dependent on the concentration of sodium sorbate or sodium benzoate; and there were considerable variations in germination inhibition among strains of *C. perfringens* (Alnoman et al., 2015). ***3.2.***

***The inhibitory activity of EOC on spore outgrowth of* C. perfringens**

The inhibitory activity of cinnamaldehyde, eugenol, and carvacrol on germination of spores of *C. perfringens* isolates led us to hypothesize that these EOC might also inhibit spore outgrowth, as previously reported with potassium sorbate, sodium benzoate and chitosan (Alnoman et al., 2015; Alnoman et al., 2017). On the other hand, the lack of inhibition of AITC on germination of *C. perfringens* spores prompts us to speculate that AITC might exert its inhibitory activity during the stage of spore outgrowth, as previously found with nisin (Udompijitkul et al., 2012). In rich TGY medium (pH 7.0), the initiation of outgrowth was detected in spores of FP strain SM101 and NFB strain F4969 after incubation for ~90 and 110 min, respectively. However, all tested EOC at a concentration of 0.05% ($p > 0.05$) significantly inhibited spore outgrowth of both strains (Fig. 1 and 2) with the exception of eguenol (Fig. 1B). Spore outgrowth inhibition was dependent on EOC concentrations (Table 2): higher inhibition was observed when cinnamaldehyde concentration was increased to 0.1%; and eugenol at a concentration of 0.1% inhibited the outgrowth of SM101 spores compare with 0.05% (Fig1 B). When the spore outgrowth of other FP isolates (E13 and NCTC 8239) was tested at eugenol concentrations 0.05% and 0.1%, significant inhibition was observed as compared to control (Table 2). In contrast, outgrowth of spores of NFB strains (B40 and NB16) were also inhibited in TGY medium supplemented with either 0.05% or 0.1% of eugenol (Table 2). Interestingly, even though AITC (0.05% and 0.1%) was not able to inhibit the spore germination, it completely blocked the outgrowth of spores FP and NFB strains (Fig. 1, 2 C, and

Table 2). Moreover, a lower concentrtaion of AITC (0.025%) exhibited significant inhibition of spore outgrowth for all strains tested (data not shown). These results are in consistent with our previous finding that nisin was able to inhibit outgrowth but not germination of spores *C. perfringens* FP and NFB isolates (Udompijitkul et al., 2012). Interestingly, carvacrol was the most effective inhibitor for germination and outgrowth of both FP and NFB spores ($p > 0.05$) (Table 2). In summary, cinnamaldehyde, AITC, and carvacrol inhibited the spore outgrowth at concentrations of 0.05% and 0.1%, however, eugenol at 0.05%, slightly inhibits the spore outgrowth of FP (E13 and NCTC 8239) and NFB (F4969, B40 and NB16) strains, but did not inhibit the outgrowth SM101 spores.

3.3. The effects of EOC on the growth of C. perfringens *vegetative cells in laboratory media*

As our results showed EOC significantly inhibited outgrowth of *C. perfringens* spores at 0.05 and 0.1%, the vegetative growth was examined in TGY medium supplemented with 0.05 and 0.1% of EOC. In case of FP SM101, cinnamaldehyde at both concentrations was able to arrest the growth of vegetative cells after 5 h of incubation, and that inhibition was continued up to 24 h (Fig. 3 A). In contrast, NFB F4969 showed greater sensitivity to cinnamaldehyde at early stage of incubation (Fig. 4A). Eugenol at a lower concentration (0.05%) illustrated no noticeable inhibition on

SM101 growth for 8 h and then resumed to a similar rate of increase as control after 24 h of incubation (Fig 3B, and Fig. 4B). However, when the concentration of eugenol was increased to 0.1%, significant growth inhibition of FP SM101 was obtained after 8 h incubation (Fig. 3B). NFB F4969 strain growing in TGY supplemented with 0.05% eugenol behaved as a control after 6 h, and then the growth started to decrease as opposed to the control. Significant inhibition of the growth of F4969 was detected at 0.1% of eugenol (Fig. 4B). Interestingly, 0.1% of AITC and carvacrol completely blocked the growth of both SM101 and F4969. No initial growth of both strains was detected, and that arresting continued for up to 24 h. A lower concentration (0.025% %) of AITC and carvacrol also exhibited significant inhibition of vegetative growth of both strains after incubation at 37^{o}C for 24 h (Fig. 3C, Fig. 4C, Fig . 3D, and Fig. 4D) and also for 48 h (data not shown). Collectively, among the four tested EOC, AITC and carvacrol were the most effective against all tested *C. perfringens* isolates.

3.4. The antimicrobial activity of EOC on spore germination and outgrowth of C. perfringens *in cooked meat product*

After noticing a significant inhibition of spore outgrowth and vegetative growth of *C. perfringens* in the presence of carvacrol and AITC in laboratory condition, we evaluated these EOC in cooked chicken meat stored at the abusive condition as previously reported (Alnoman et al., 2015). Results revealed that AITC successfully

inhibited the growth of both FP and NFB spores in chicken meat during 6 h storage time under anaerobic condition at 37 ^{o}C. After 6 h incubation, a dose of 0.5 % AITC was sufficient to inhibit the growth of spores which resulted less growth of viable cell count by more than 1 log. As AITC concentration increased to 1.5 - 2 %, significant inhibition of FP and NFB spores were observed and resulted in more than 2 log less growth compared to the positive control (Fig. 5). These data suggested that the antimicrobial activity of AITC against spores of FP and NFB isolates is concentration dependent.

On the other hand, none of the tested concentrations (0.5 - 2.5%) of carvacrol was able to inhibit the spore growth after 6 h of incubation in chicken meat system. Even at a very high concentration (5 %), carvacrol showed no significant inhibition of spore growth in chicken meat (Fig. 5B). These results are contradictory to the results found in a previous report (Nair et al., 2014), *Salmonella spp.* was inhibited by 1% of carvacrol in turkey breast cutlets. Furthermore, meat samples containing cinnamaldehyde and eugenol at various concentrations exhibited no inhibition of spore growth in chicken meat after 6 h incubation at 37 ^{o}C under anaerobic condition (data not shown). Collectively, our resuts indicate that among four tested EOC, only AITC was able to inhibit the growth of cocktail spores of *C. perfringens* FP and NFB isolates in chicken meat model. AITC is the constituent of mustard and garlic oil and widely used by the food industry as a flavoring agent and is classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the

United States (Hyldgaard et al., 2012). Previous reports have shown the inhibitory activity of AITC against various food spoilage microorganisms and food pathogens including *E. coli* O157:H7, *S. Typhimurium*, *L. monocytogenes*, and other Gram-negative aerobic spoilage bacteria (LIN, JAMES F. PRESTON III, & WEI, 2000). The action behind AITC's antimicrobial activity is not well understood, however, it has been shown to cause cell wall damage to *L. monocytogenes* and that confirmed by transmission electron microscopy (TEM) (LIN et al., 2000). Another study has indicated that AITC caused cell membrane damage to *E. coli* and *Salmonella spp* (Piercey et al., 2012). Further research on mechanism of AITC mediated growth inhibition of *C. Perfringens* should answer whether similar cell-demaged mecahsism is possible for *C. Perfringens*.

***Conclusions*:**

This study demonstarted the following important findings: (1) cinnamaldehyde, eugenol, and carvacol but not AITC exhibited noticeable inhibitory activity against spore germination of *C. perfringens* FP and NFB isolates. (2) all tested EOC showed inhibitory activity against spore outgrowth and vegetative growth of *C. perfringens* FP and NFB isolates, especially AITC and carvacrol, their inhibitory activity were shown at lower concentrations. (3) NFB isolates was more sensitive to EOC compared to the FP. (4) the inhibitory activity of AITC was observed against spore growth of *C. perfringens* FP and NFB isolates inoculated into a chicken meat. however, no inhibition of *C. perfringens* growth were observed in chicken meat supplemented with carvacrol. The inhibitory activity of AITC against spores of *C. perfringens* on chicken meat was detected at concentration range from 0.5 to 1% and significant inhibition was shown on viable cell count at 1.5 and 2%. The antimicrobial agents used in this study are generally recognized as a safe (GRAS) agents to food formulation. Combination of these EOC should be an active method to control the risk of *C. perfringens* FP and NFB diseases.

Figures

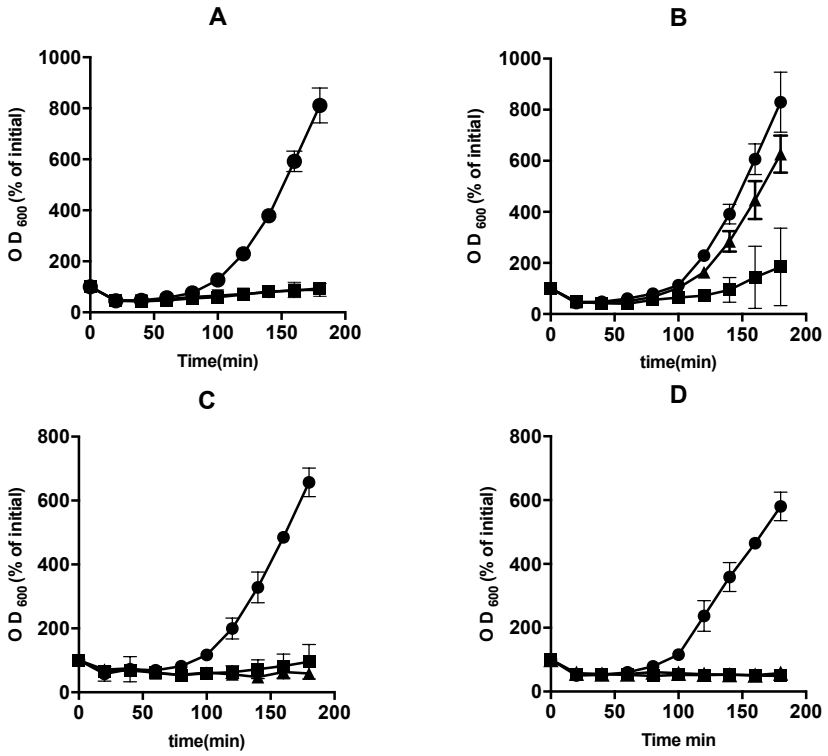

Fig. 1. Effects of EOC on outgrowth of spores of FP strain SM101. Spores of *C. perfringens* FP strain SM101 were heat-activated and inoculated into fresh TGY broth supplemented with cinnamaldehyde (A), eugenol (B), AITC (C), and carvacrol (D). Outgrowth of spores was measured as described in Material and methods. Symbols represent concentrations: circles, TGY only; squares, TGY plus 0.05 %; EOC and triangles, TGY plus 0.1% EOC. Each data point represents an average of triplicate experiments with three independent spore preparations. Error bars represent the standard deviation of the mean.

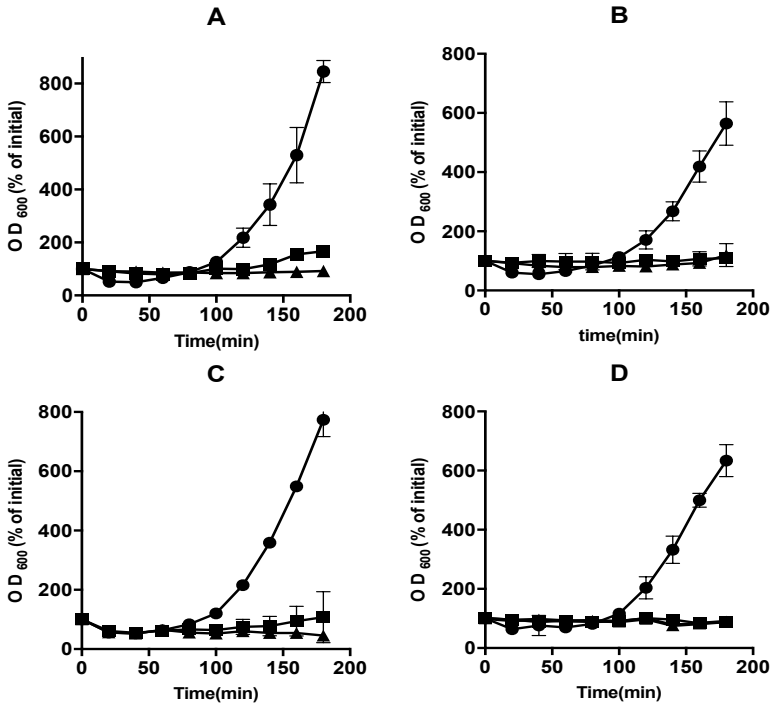

Fig. 2. Effects of EOC on outgrowth of spores of NFB strain F4969. Spores of *C. perfringens* NFB strain F4969 were heat- activated and inoculated into fresh TGY broth supplemented with cinnamaldehyde (A), eugenol (B), AITC (C), and carvacrol (D). Outgrowth of spores was measured as described in Material and methods. Symbols represent concentrations: circles, TGY only; squares, TGY plus 0.05 % EOC; and triangles, TGY plus 0.1% EOC. Each data point represents an average of triplicate experiments with three independent spore preparations. Error bars represent the standard deviation of the mean.

plus (0.1% essential oils). Error bars represent the standard deviation of the mean of three replicates.

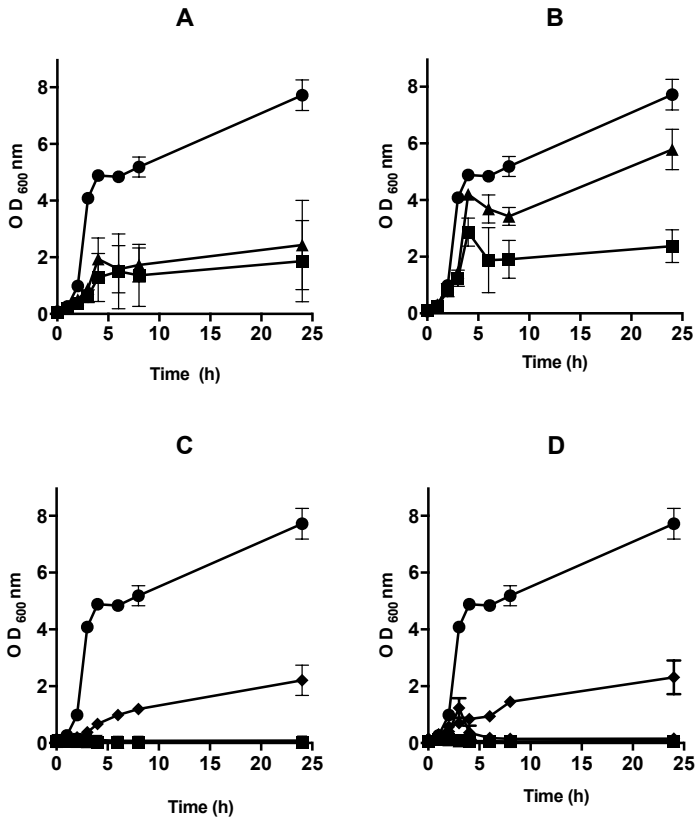

Fig. 3. Effects of EOC on vegetative growth of FP strain SM101. Vegetatively growing (3-h TGY grown culture) cells of FP strain SM101 were inoculated into TGY medium containing various concentrations of cinnamaldehyde (A), eugenol (B), AITC (C), or carvacrol (D) and growth was monitored by measuring OD_{600} at hourly intervals. Symbols represent concentrations: circles TGY only, diamonds, 0.025% %; triangles, 0.05%; and squares, 0.1%. Error bars represent the standard deviations of the mean of three replicates.

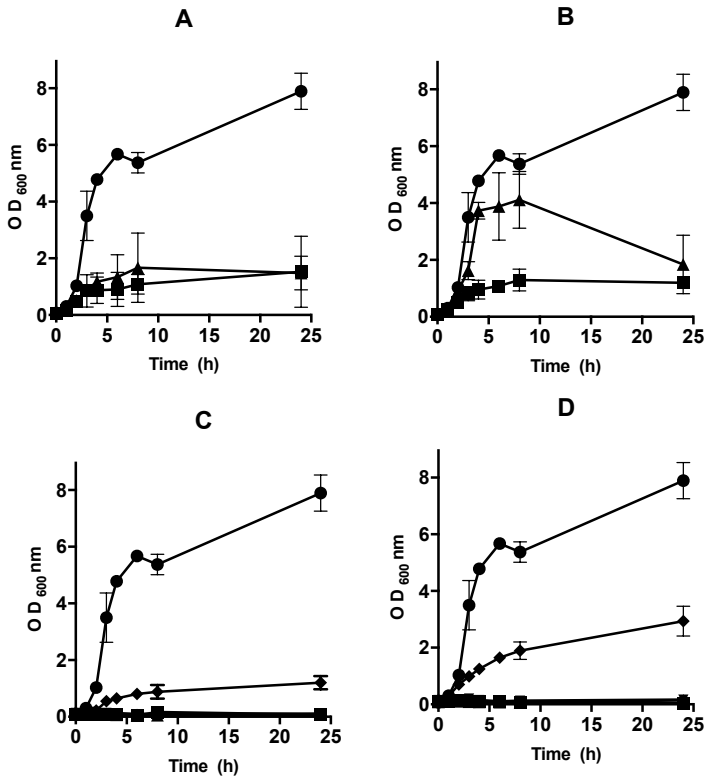

Fig. 4. Effects of EOC on vegetative growth of NFB strain F4967. Vegetatively growing (3-h TGY grown culture) cells of NFB strain F4969 were inoculated into TGY medium containing various concentrations of cinnamaldehyde (A), eugenol (B), AITC (C), or carvacrol (D) and growth was monitored by measuring OD_{600} at hourly intervals. Symbols represent concentrations: circles, TGY only, diamonds, 0.025 %; triangles, 0.05%; and squares, 0.1%. Error bars represent the standard deviations of the mean of three replicates.

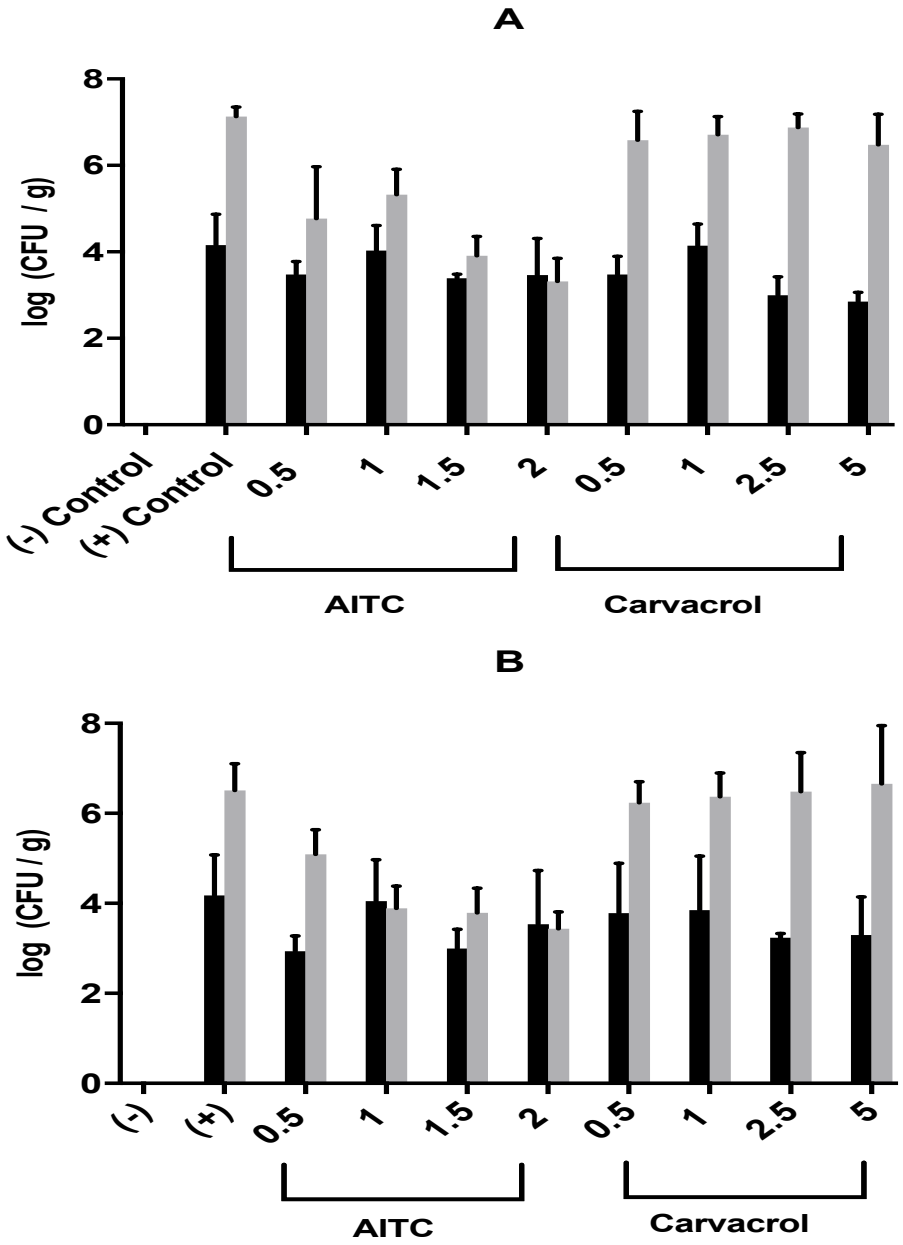

Fig. 5. Effects of EOC on growth of *C. perfringens* spores in cooked chicken meat. Cocktail spores of 3-strain of *C. perfringens* FP (A) or NFB (B) isolates were inoculated into cooked chicken samples containing different concentrations of AITC or carvacrol as indicated. CFU formed by survival spores were determined by plating onto BHI agar and incubating anaerobically at 37°C for 24 h. Black bars, initial CFU, and gray bars, viable CFU in cooked chicken samples after 6 h of anaerobic incubation at 37 °C. Error bars represent standard deviation from the mean of three independent experiments.

Tabels

Table 1: Inhibition of *C. perfringens* spore germination by EOC[a]

Inhibitor	Concentration (% v/v)	Inhibition of germination (% ± SD)[b]					
		FP strains			NFB strains		
		SM101	E13	NCTC8239	F4969	B40	NB16
Cinnamaldehyde	0.05	4 ± 0.5	38 ± 5.5	28 ± 14.2	74 ± 3.0	11 ± 1.5	39 ± 14.0
	0.1	35 ± 5.5	51 ±11.2	62 ± 10.4	77 ± 6.0	45 ±15.0	55 ± 11.0
Eugenol	0.05	5 ± 5.6	27 ± 1.0	37 ± 21.0	93 ± 2.5	12 ± 3.0	24 ± 1.5
	0.1	18 ± 3.2	65 ± 3.0	52 ± 11.0	99 ± 1.5	33 ± 6.13	67 ± 17.0
AITC[c]	0.05	0.6 ± 2.9	9 ± 0.9	4 ± 1.4	6 ± 11.4	2 ± 6.0	2 ± 3.1
	0.1	0.2 ± 3.1	12 ± 1.0	5 ± 1.6	22 ± 7.0	3 ± 5.6	4 ± 3.4
Carvacrol	0.05	50 ± 7.9	43 ± 15.4	51 ± 13.4	100 ± 3.0	22 ± 5.1	87 ± 11.0
	0.1	64 ± 3.2	66 ± 7.3	65 ± 11.8	100 ± 1.0	90 ± 11.0	100 ± 3.0

[a] Spore were germinated in TGY containing various concentration of different essential oils as indicated, and percentage of inhibition of germination was calculated as described in Material and method.

[b] Values are the average of triplicate experiments with two different spore preparation. SD represents standard deviation of the mean.

[c] AITC indicates Allyl isothiocyanate.

Table 2: Inhibition of *C. perfringens* spore outgrowth by EOC[a]

Inhibitor	Concentration (% v/v)	Inhibition of outgrowth (% ± SD)[b]			
		FP strains		NFB strains	
		E13	NCTC8239	B40	NB16
Cinnamaldehyde	0.05	76 ±8.3	60 ±1.3	76±10.0	79±17.0
	0.1	85 ±9.1	84 ±15.0	78±15.0	87±6.7
Eugenol	0.05	61 ±11.3	60 ±2.8	70±13.0	55±5.9
	0.1	78 ±9.8	76 ±2.2	84±3.0	71±10.5
AITC[c]	0.05	92 ±5.0	91 ±9.0	94±10.8	90±2.7
	0.1	88 ±7.7	83 ±14.8	87±11.0	91±2.0
Carvacrol	0.05	90 ±2.0	86 ±17.0	98±13.0	80±15.0
	0.1	91 ±10.0	86 ±18.0	99 ±9.9	83±15.0

[a] Heat-activated spores were inoculated into TGY medium supplemented with different essential oils at various concentrations as indicated. Spore outgrowth was assessed after 3 h of incubation at 37°C and inhibition of outgrowth was calculated as described in Material and methods.

[b] Values are the average of triplicate experiments with two different spore preparation. SD indicates standard deviation of the mean.

[c] AITC indicates Allyl isothiocyanate.

Chapter 3

General Conclusion

Clostridium perfringens is a gram-positive, anaerobic, rod-shaped bacterium that causes *C. perfring*ens type A FP and NFB GI diseases in human and other animal (Labbe & Tang, 1983; Li et al., 2013). The ubiquitousness of this bacteria made broad chances to contaminate food products. The pathogenicity of *C. perfringens* largely results from its spores' resistance ability (B. A. McClane & Chakrabarti, 2004; Smedley et al., 2004). When the spores of *C. perfringens* contaminate food, spores possess enough heat resistance to survive food processing treatment causing food poisoning diseases (Awad & Rood, 1997; Mead et al., 1999). Instead of killing these spores, the heat encourages the dormant spores to germinate to become active cells and quickly multiply reaching dangerous level that can cause foods contamination (Cordoba et al., 2001; Sarker et al., 2000). Basically, most of the outbreaks of *C. perfringens* type A FP are associated with meat and poultry products (B. A. McClane & Chakrabarti, 2004). Developing inactivation strategies to prevent the growth of *C. perfringens* in food products is important for meeting consumer demands which are safe, minimally-processed, convenient, and ready to eat food product. Using natural antimicrobial agents in food products is required to prolong the shelf-life of foods by limiting growth and survival of spores and vegetative cells of *C. perfringens*.

In the present work, the antimicrobial activity of EOC (cinnamaldehyde, eugenol, carvacrol, Allyl isothiocyanate AITC) has been determined in laboratory

conditions and meat model system against *C. perfringens* FP and NFB isolates. In laboratory conditions, results showed that cinnamaldehyde, eugenol, and carvacrol but not AITC inhibited the spore germination of FP and NFB isolates. However, all tested EOC exhibited significant inhibition of spore outgrowth. During vegetative growth, carvacrol and AITC completely blocked the growth of *C. perfringens* FP and NFB strains. Finally, cinnamaldehyde, eugenol, and carvacrol showed no inhibitory activity in chicken meat model. However, only AITC was able to inhibit the growth of *C. perfringens* in chicken meat and this inhibition was dependent on AITC concentration. Collectively, our current work contributes to food industry in order to control *C. perfringens* spores and vegetative cells of both FP and NFB isolates in laboratory condition and chicken meat model system. The antimicrobial agents used in this study are generally recognized as a safe (GRAS) agents to food formulation. Combination of these oils components should be an active method to control the risk of *C. perfringens* FP and NFB diseases.

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